Supplementary Figure 1-12 and SI Text for "β-amyloid and α-synuclein cooperate to block SNARE-dependent vesicle fusion"

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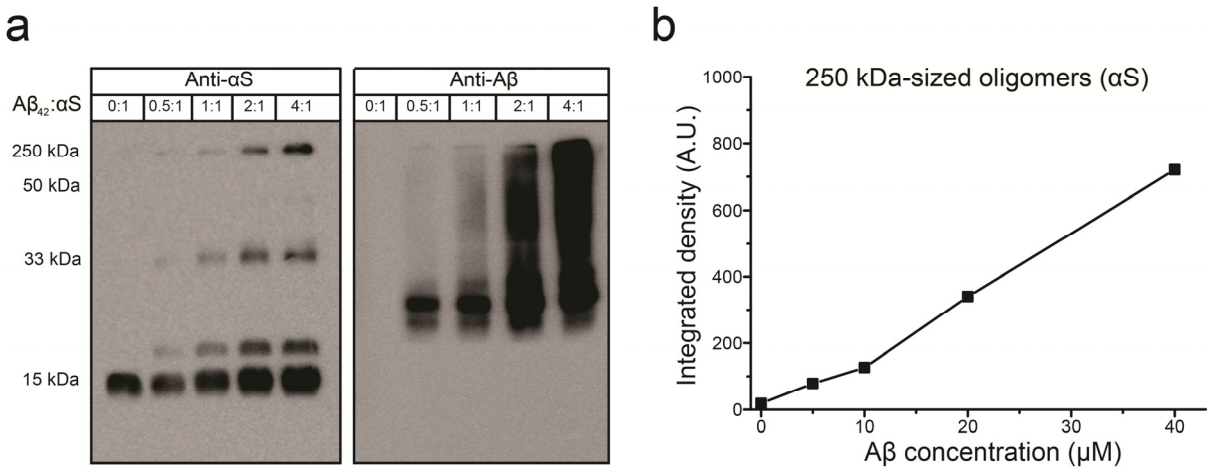
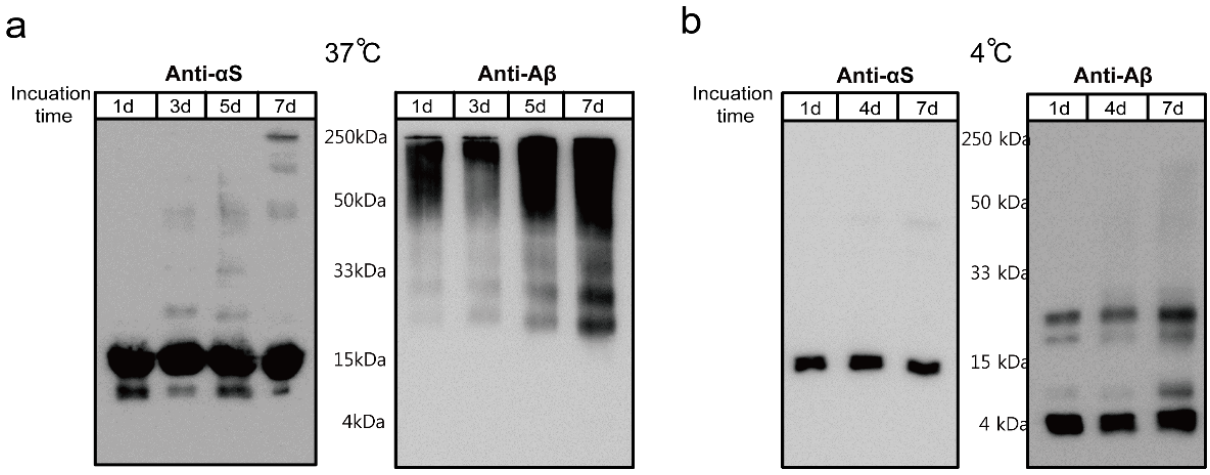
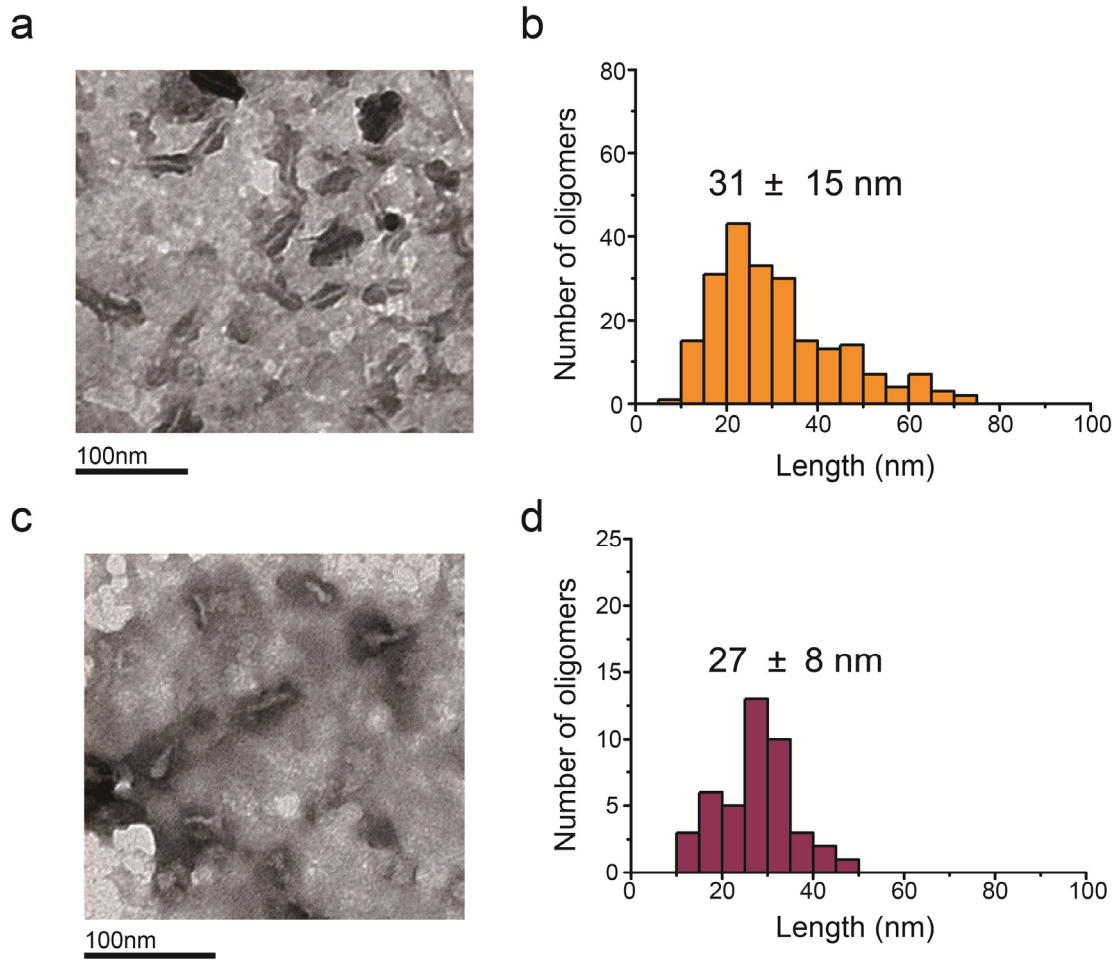


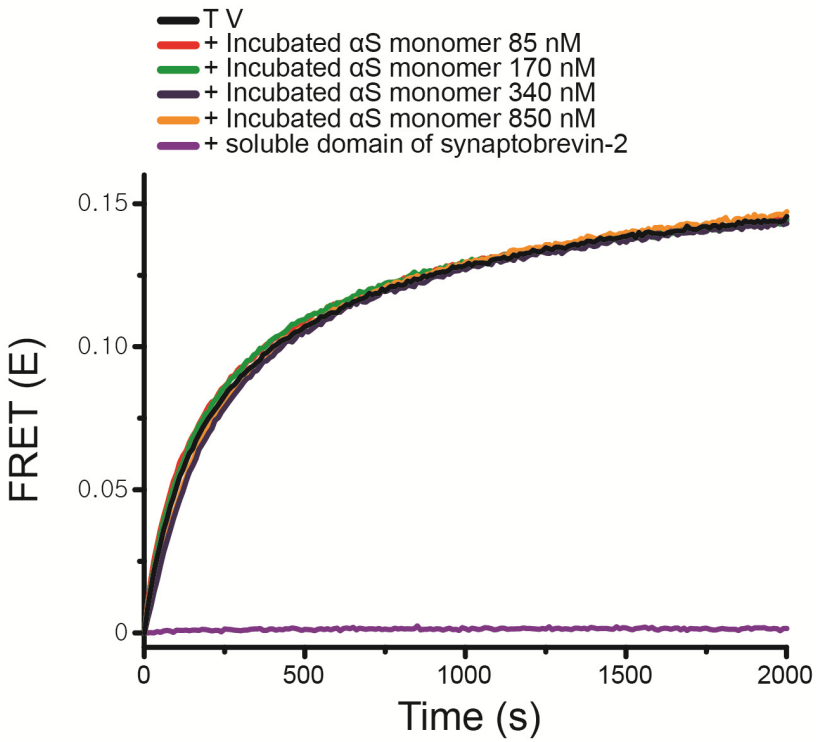
Supplementary Figure 1. Concentration titration test of Aβ-seeded αS oligomers. (a) 10 μM αS oligomers were incubated with no (0:1), 5 μM (0.5:1), 10 μM (1:1), 20 μM (2:1), and 40 μM (4:1) Aβ for 7 days at 37 °C. Aβ-seeded αS oligomers were detected by αS-antibody (left) and Aβ-antibody (right). 250 kDa-sized Aβ-seeded αS oligomers were detected at the ratio of 0.5:1 and amounts of large Aβ-seeded αS oligomers increased in a dose-dependent manner. (b) Quantification of 250 kDa-sized Aβ-seeded αS oligomers based on the band intensities from (a).



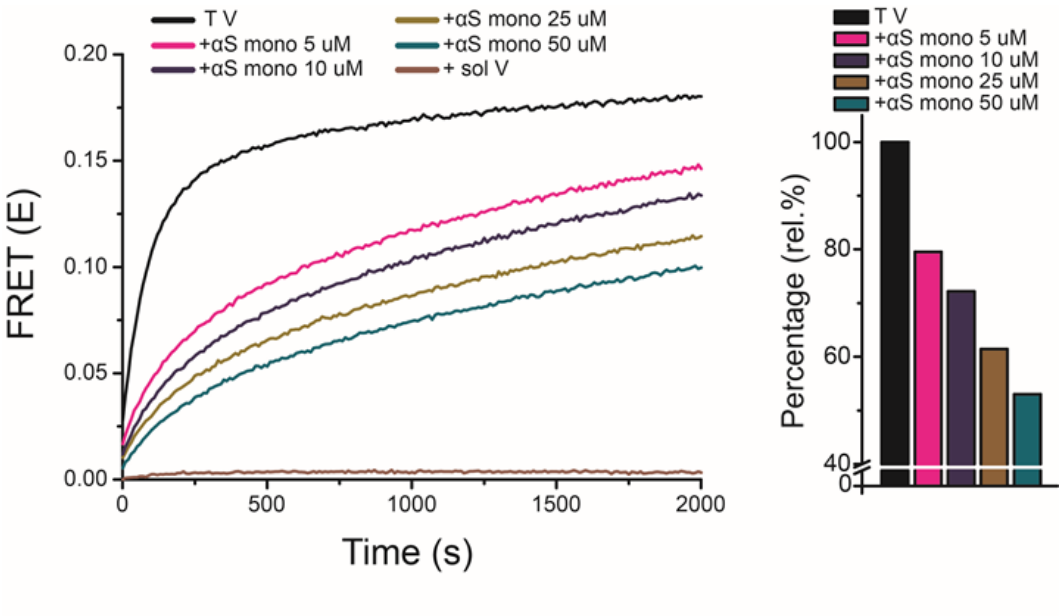
Supplementary Figure 2. Time-dependent products of the mixture of Aβ and αS at 4 °C and 37 °C. 10 μM αS and 20 μM $A\beta_{42}$ monomers were incubated in 20 mM sodium phosphate buffer at 37 °C (a) and 4 °C (b). Western blots of the unpurified mixture were obtained at various time points.



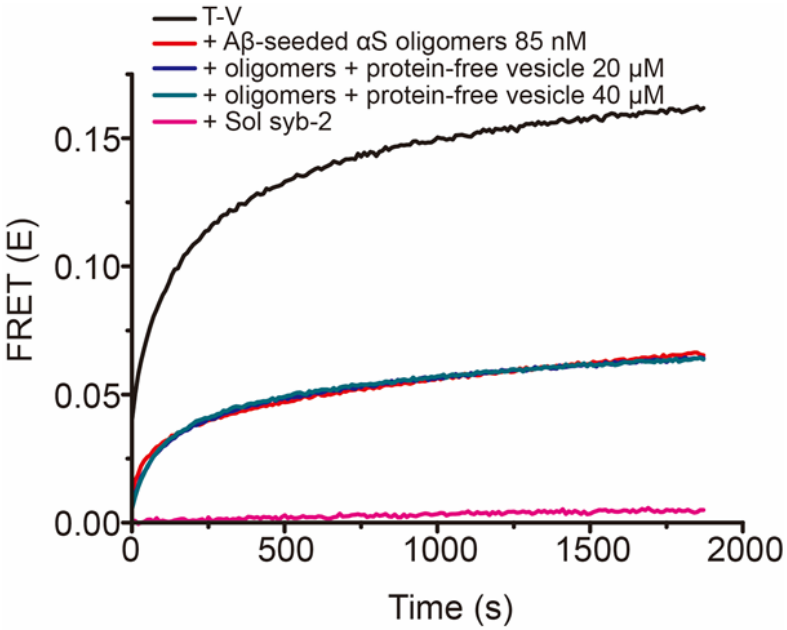
Supplementary Figure 3. Morphological study of Aβ-seeded αS oligomers and cross-linked αS oligomers by TEM. (a) Representative TEM image of Aβ-seeded αS oligomers. About 79 % of oligomers show rod-like shape and the rest of oligomers show annular form. (b) Length distribution of rod-like shape oligomers. The mean is approximately 31 nm. (c) Representative TEM image of cross-linked αS oligomers. Similar to Aβ-seeded αS oligomers, cross-linked αS oligomers have mostly rod-like shape structure (75 %). (d) Length distribution of rod-like shape oligomers. The mean of length is approximately 27 nm.



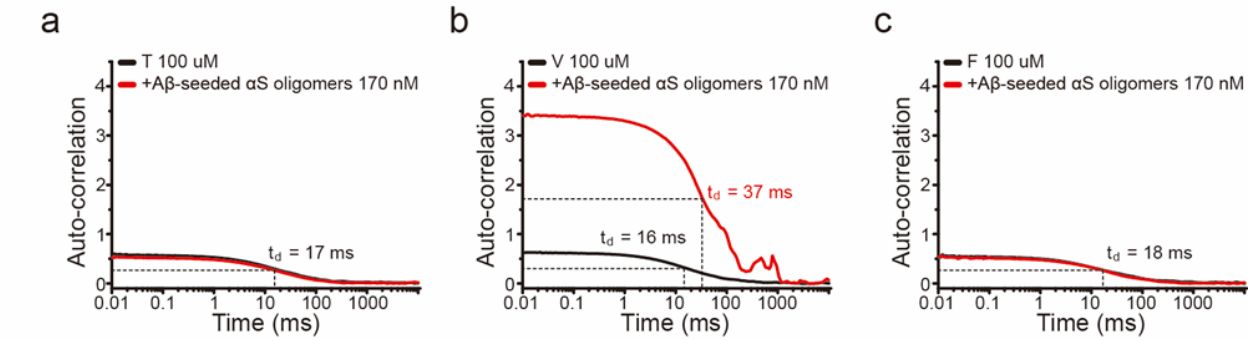
Supplementary Figure 4. Effects of the products of αS incubated without Aβ$_{42}$ on SNARE-mediated vesicle fusion. T-V: **T** and **V** (20 μM in lipid concentration) were mixed together at 35 °C without any additives (black line). Negative lipid mixing control was obtained by adding a soluble motif of synaptobrevin-2 (purple line). When the products of αS incubated without Aβ$_{42}$ were added to the **T-V** fusion reaction, no significant reduction in lipid mixing was observed (85 nM, red line, 170 nM, green line; 340 nM, dark blue line; and 850 nM, orange line [αS monomer concentration]).



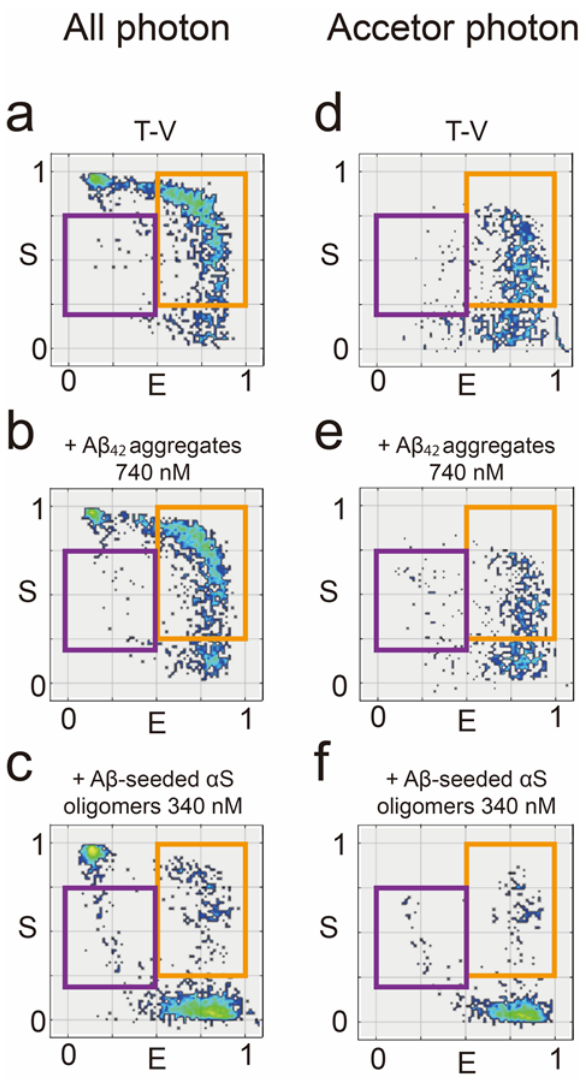
Supplementary Figure 5. Effect of a high concentration of αS monomer on ensemble lipid mixing. We conducted ensemble lipid mixing assays in the presence of high concentrations of αS oligomers, ranging from 5 μM to 50 μM, to examine the effect of high concentrations of αS oligomers on lipid mixing. The lipid mixing was significantly reduced by the high concentration of αS monomer. The right bar graph indicates the relative percentage of lipid mixing at 1800 s from the data of the graph in the left panel.



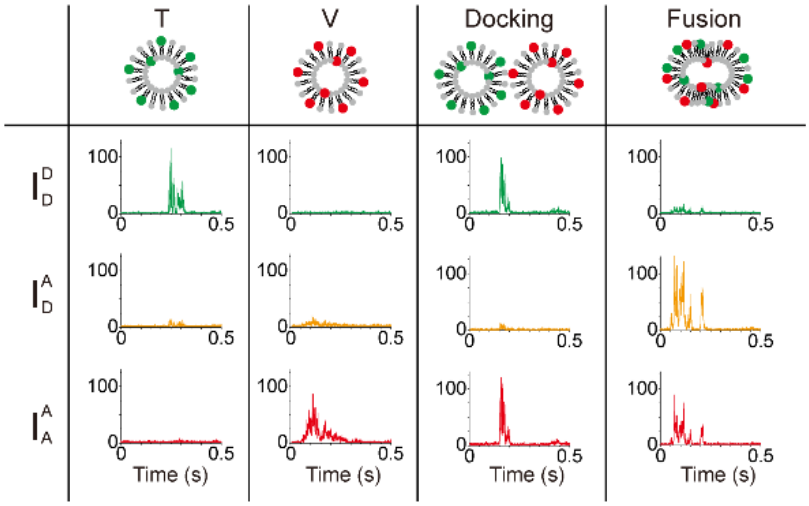
Supplementary Figure 6. Effect of negatively charged liposomes on lipid mixing in the presence of Aβ-seeded αS oligomers. We tested whether Aβ-seeded αS oligomers bind to negatively charged lipids by adding excess amounts of protein-free liposome containing 15 % PS to the bulk lipid mixing assay. We added 20 μM or 40 μM (lipid concentration) protein-free liposome to the fusion reaction (20 μM total lipid concentration) in the presence of Aβ-seeded αS oligomers. If Aβ-seeded αS oligomers bind to negatively charged liposomes, the oligomers would bind to protein-free liposomes, which should reduce the concentration of free Aβ-seeded αS oligomers in the fusion mixture compared with the case of no protein-free liposomes. Thus, the addition of protein-free liposomes would dampen the fusion inhibitory effects of Aβ-seeded αS oligomers (i.e., increase the fusion efficiency). However, no change in the fusion efficiency was observed with and without protein-free liposomes in the presence of Aβ-seeded αS oligomers.



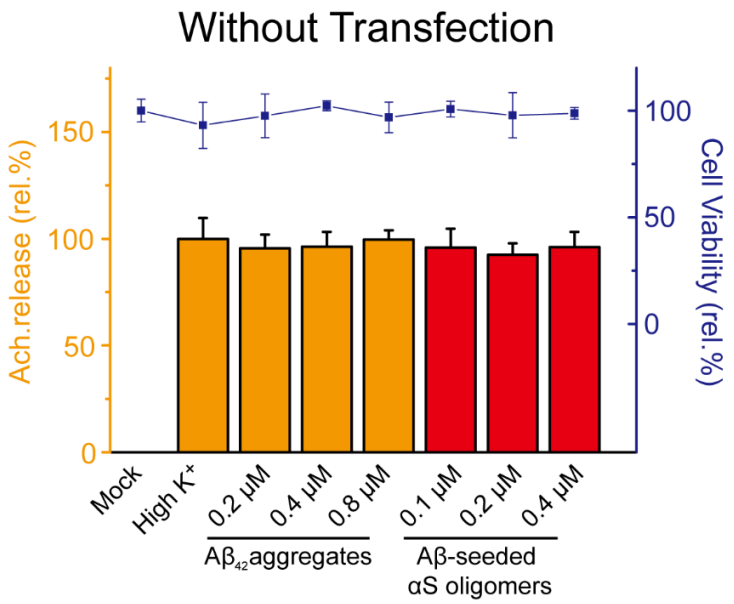
Supplementary Figure 7. Fluorescence correlation spectroscopy (FCS) measurement of vesicle clustering by Aβ-seeded αS oligomers. To examine whether Aβ-seeded αS oligomers bind to and cluster proteoliposomes, we conducted FCS measurements. The autocorrelation curves of (a) t-vesicles (**T**), (b) v-vesicles (**V**), and (c) protein-free liposomes (**F**) labeled with 2 % DiI were obtained from FCS measurements in the presence (red line) and absence (black line) of 170 nM Aβ-seeded αS oligomers. FCS measures the diffusion times (t_d) of vesicles in buffer solution, and these times correspond to the size of vesicles. Clustering significantly increases the diffusion time of vesicles. The diffusion times of t-vesicles were nearly identical, approximately 17 ms, in both cases; this finding indicates that the Aβ-seeded αS oligomers did not induce clustering of the t-vesicles. Similarly, Aβ-seeded αS oligomers did not induce clustering of protein-free liposomes. In contrast, the diffusion times of v-vesicles, which were reconstituted with VAMP-2, were significantly increased (37 ms) in the presence of Aβ-seeded αS oligomers. These results demonstrate that Aβ-seeded αS oligomers specifically cluster v-vesicles; this clustering is mediated by the interaction between v-SNARE and Aβ-seeded αS oligomers.



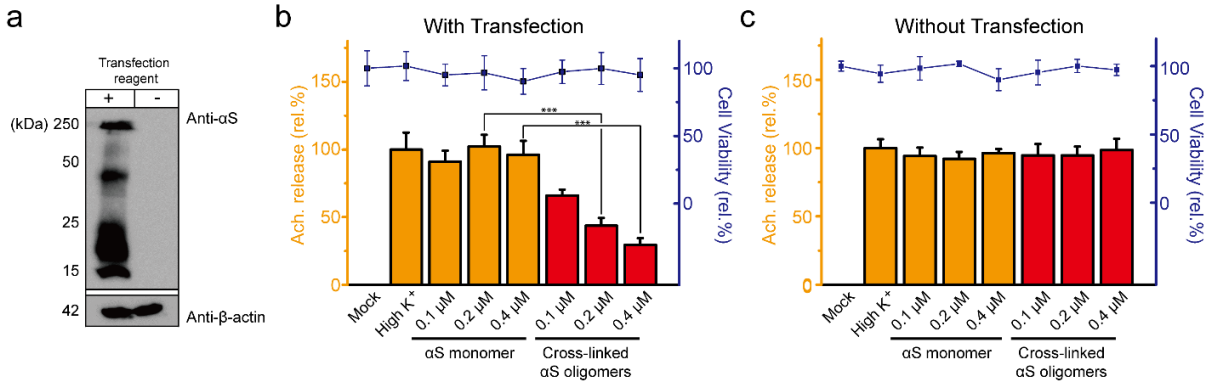
Supplementary Figure 8. Comparison of two burst search methods. 2D E-S graphs of (a) T-V (the same as Figure 3e), (b) T-V in the presence of 740 nM Aβ aggregates (the same as Figure 3g), and (c) T-V in the presence of 340 nM Aβ-seeded αS oligomers (the same as Figure 3f) generated by all photon search methods. 2D E-S graphs of (d) T-V, (e) T-V in the presence of 740 nM Aβ aggregates, and (f) T-V in the presence of 340 nM Aβ-seeded αS oligomers generated by acceptor photon search methods. The all photon search is used to present all vesicles in solution. However, the all photon search method cannot quantify subpopulations of vesicles because t-vesicles, v-vesicles and fused-vesicles are not selected equally. Thus, quantification of the sub-populations of vesicles from an E-S graph was performed by the acceptor photon search.[1,2]



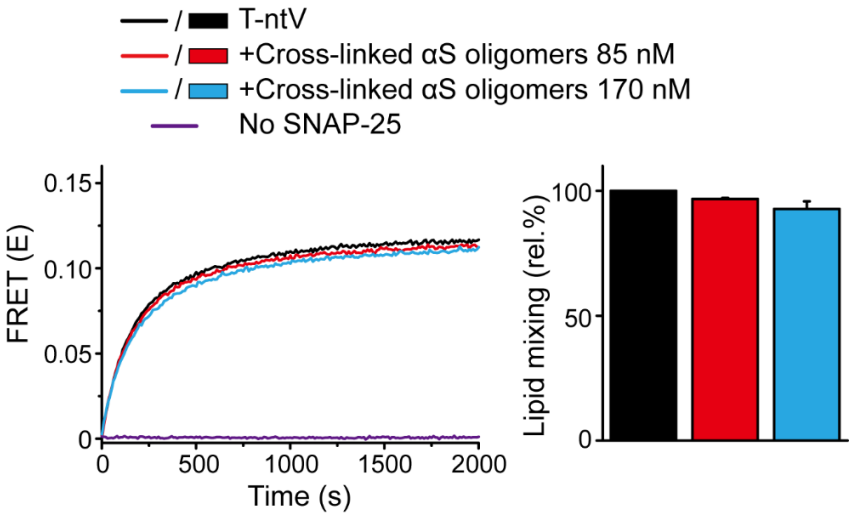
Supplementary Figure 9. Time traces of the single vesicle fusion assay measured by ALEX. Typical fluorescence time traces of vesicles obtained by ALEX. Sufficient dilution to 100 pM vesicles ensures that only one vesicle passes through the excitation volume at a given time. The detected fluorescent emissions result in fluorescence time traces. ALEX generates three types of fluorescence emissions for a vesicle: I^D_D, the emission of DiI (donor dye) excited by the donor-excitation laser (green line); I^A_D, the emission of DiD (acceptor dye) excited by the donor-excitation laser, which is a FRET signal (orange line); and I^A_A, the emission of the acceptor dye excited by the acceptor-excitation laser (red line). Depending on the reaction status, unreacted **T** and **V**, docked, and lipid-mixed vesicles have different values for the three fluorescence intensities.



Supplementary Figure 10. Controls without transfection reagents for Figure 4. Error bars were generated from three independent measurements



Supplementary Figure 11. Effects of chemically cross-linked αS oligomers on PC12 cell exocytosis. We tested the effects of chemically cross-linked αS oligomers on PC12 cell exocytosis, as in Figure 4, using Aβ-seeded αS oligomers. (a) The transfection of chemically cross-linked αS oligomers into PC12 cells was confirmed by western blot. Cell lysates were analyzed by western blot analysis and probed with an anti-αS antibody (top panel). The amount of protein loaded was confirmed visualizing the β-actin levels (bottom panel). In the absence of transfection reagent, no chemically cross-linked αS oligomers were detected. (b) The effects of chemically cross-linked αS oligomers delivered into the PC12 cells on exocytosis. After delivering the chemically cross-linked αS oligomers into PC12 cells by transfection, the amount of released [^{14}C]-acetylcholine after a high-K^+ depolarization was measured (***P<0.005). An MTT assay was performed to ensure cell viability after transfection. (c) Controls without transfection reagents. The error bars were obtained from three independent measurements.



Supplementary Figure 12. The effects of cross-linked αS oligomers on vesicle fusion reconstituted with nt-synaptobrevin-2. No inhibitory effects on the **T-ntV** fusion mixture were observed. Bar graphs were obtained from three independent measurements.

SI Text

Preparation of cross-linked αS oligomers. To prepare αS oligomers, 25 μM αS was incubated with 500 μM BS^3 (Bis[sulfosuccinimidyl] suberate, Thermo) cross-linker in 20 mM sodium phosphate buffer (pH 7) at room temperature for 1 hour. Then, 25 mM Tris-HCl was added to the mixture and incubated at room temperature for 15 min to quench the BS^3. The sample was purified by size exclusion chromatography (SEC) using a superdex^{TM}200 10/300GL (GE healthcare) and concentrated using an Ultracel 10k-membrane.

Preparation of SNARE proteins and reconstituted proteoliposomes. His6×-tagged syntaxin-H_{abc}-truncated (syntaxin-HT, 168-288 a.a., two Cys were replaced with Ala) and full-length Synaptotagmin-1 (1-421 a.a.) were inserted into the pET28-a vector and purified using a Ni-NTA resin column. GST-tagged Syntaxin-1A full-length (1-288 a.a., 3 Cys were replaced with Ala), SNAP-25 (synaptosomal-associated protein-25, 1-206 a.a., four Cys were replaced with Ala), synaptobrevin-2 (VAMP-2, 1-116 a.a., one Cys was replaced with Ala), and N-terminal truncated mutant of synaptobrevin-2 (nt-synaptobrevin-2, 29-116 a.a.) were inserted into the pGEX-KG vector and purified using a glutathione-agarose resin column. BL21 Rosetta (DE3) pLysS cells (Novagene) were used to express all proteins. To obtain artificial proteoliposomes, synthetic lipid components, POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), DOPS (1,2-dioleoyl-sn-glycero-3-[phospho-L-serine]), cholesterol, and $PI(4,5)P_2$ (1,2-dioleoyl-sn-glycero-3-phospho-[1'-myo-inositol-4',5'-bisphosphate]) were purchased from Avanti Polar Lipid. In the lipid mixing assay, lipid analogue fluorescent dyes, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI, donor dye, Invitrogen) and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD, acceptor dye, Invitrogen) were used. The following lipid ratios were used for liposome formation: POPC:DOPS:Chol:DiI (71:7:20:2) for t-vesicles (**T**) and POPC:DOPS:Chol:DiD (71:7:20:2) for v-vesicles (**V**). The lipid mixtures were dried and re-suspended in vesicle buffer (25 mM HEPES, 100 mM KCl, pH 7.4), and extrusion was performed to make monodisperse unilamellar vesicles using a mini extruder (Avanti Polar Lipid) with a 100-nm polycarbonate filter (Whatman).

Binary t-SNAREs were pre-formed by incubating syntaxin-1A (or Syntaxin-HT) and SNAP-25 at a 1:2 molar ratio at room temperature for 1 hour (Syntaxin-HT was used for **T-V** fusion, and Syntaxin-1A was used for **T-SV** fusion). Binary t-SNAREs, extruded vesicles, and Octyl β-D-glucopyranoside (O.G.) detergent were mixed together at 0.7 % (w/v) of the final O.G. concentration to prepare t-vesicles. The v-SNARE VAMP-2, extruded vesicles, and O.G. detergent were mixed together at 0.8 % (w/v) of the final O.G. concentration to prepare v-vesicles. For **T-V** lipid mixing, the lipid-to-protein molar ratio (L/P) was 200:1 for t-SNARE and v-SNARE. For the content mixing assay, sytaptotagmin-1 was added to v-vesicles (**SV**). The L/P was 500:1 for t-SNARE and v-SNARE, and 900:1 for synaptotagmin-1 in the content mixing assay. Protein-lipid-detergent mixtures were dialyzed at 4 °C overnight (Slide-A-Lyzer mini dialysis kit, Thermo) to remove the O.G. detergent. The following lipid ratios were used: POPC:DOPS:Chol:PIP_2 (72:7:20:1) for t-vesicles and POPC:DOPS:Chol (73:7:20) for v-vesicles (**SV**). Twenty millimolar sulforhodamine B (SRB, Invitrogen) was encapsulated in the v-vesicles, whose fluorescent signal was used as an indicator of content mixing. After dialysis, **SV** containing SRB was purified using a CL-4B column to remove free SRB.

In vitro* single vesicle fusion assay measured by ALEX.** The instrumental setup and data analysis procedures of alternating-laser excitation (ALEX) have been described elsewhere.[1] Briefly, for the single-vesicle lipid-mixing assay, t- and v-vesicles (20 μM final lipid concentration) were mixed and incubated for 30 min at 35 °C, using the same conditions as the bulk fusion assay in the presence or absence of Aβ-seeded αS oligomers or $A\beta_{42}$ aggregates. Incubated samples were diluted three times, and then ALEX measurements were performed (10 min of data collection). In ALEX measurements, three types of fluorescent emissions were obtained from each vesicle ($\mathbf{I^D_D}$, the fluorescent emission of the donor dye excited by the donor excitation laser; $\mathbf{I^A_D}$, the fluorescent emission of the acceptor dye excited by the donor excitation laser; and $\mathbf{I^A_A}$, the fluorescent emission of the acceptor dye excited by the acceptor-excitation laser), and these three fluorescence intensities from each vesicle were used to calculate two parameters, ***E (FRET efficiency) and ***S*** (stoichiometry ratio or sorting number). To analyze the fraction of lipid-mixed vesicles, we graphically selected vesicle numbers from the ***E-S*** graph as previously described.[2]

Measuring [^{14}C]-acetylcholine release from PC12 cells and MTT cytotoxicity assay

PC12 cells obtained from Korean Cell Line Bank were maintained at the Roswell Park Memorial Institute (RPMI) at 37 °C in a 5% CO_2 incubator. Aβ-seeded αS oligomers or $Aβ_{42}$ aggregates were transduced into PC12 cells using the Pro-Ject Protein Transfection Reagent (Thermo). Before transfection, 50 ng/ml of neuronal growth factor (NGF) was treated to PC12 cells for 5 days for differentiation. Then, differentiated PC12 cells were pre-depolarized with a high-K^+ solution (115 mM NaCl, 50 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 11 mM glucose, and 15 mM Hepes-Tris at pH 7.4) to release already-loaded neurotransmitters from vesicles for exocytosis (the readily releasable pool). After 15 min, 2.5 μl of the mixtures of the transfection reagent with various concentrations of Aβ-seeded αS oligomers, $Aβ_{42}$ aggregates, or cross-linked αS oligomers were added and incubated for 3 h at 37 °C. Then, [^{14}C]-acetylcholine (1 μCi/mL) was applied for 60 min in a low-K^+ solution (140 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$,1.2 mM $MgSO_4$, 11 mM glucose, and 15 mM Hepes-Tris at pH 7.4). The cells were washed four times to remove unincorporated radiolabeled neurotransmitters, then depolarized with a high-K^+ solution for 15 min to assess the release. Extracellular medium was transferred to scintillation vials, and the quantity of released neurotransmitters was measured by liquid scintillation counting. Cell viability was assessed using a modified MTT assay. The MTT solution was added to each well with 1/5 volume of media. After incubating for 3 h at 37 °C in the dark, the absorbance was measured at 570 nm by a multi-well plate reader. For each treatment, cell viability was calculated as a relative percentage of the un-treated control.

Co-floatation assay used to detect the binding of Aβ-seeded αS oligomers to proteoliposomes. 100 μM (lipid concentration) of **T**, **V**, **ntV** (v-vesicle reconstituted with nt-synaptobrevin-2 instead of the full-length synaptobrevin-2), and **F** (protein-free) vesicles were incubated with 170 nM Aβ-seeded αS oligomers (concentration of αS monomers) for 30 min at room temperature to a final volume of 400 μL, and then an equal volume of 80 % (w/v) Histodenz was added to the mixture to create a 40 % Histodenz solution. The mixture was transferred to a thick-wall centrifugation tube (Beckman), and 300 μL of 30 % (w/v) Histodenz and 100 μL of vesicle buffer (0% Histodenz) were added in order without mixing. A SW55Ti rotor (Beckman) was used for

gradient centrifugation at 260,000 × g for 150 min at 4°C. Vesicle-bound Aβ-seeded αS oligomers were obtained between 0 % and 30 % Histodenz, and analyzed by western blot.

References

(1) Kim, J. Y.; Choi, B. K.; Choi, M. G.; Kim, S. A.; Lai, Y.; Shin, Y. K.; Lee, N. K. *EMBO J.* **2012**, *31*, 2144.
(2) Choi, B. K.; Choi, M. G.; Kim, J. Y.; Yang, Y.; Lai, Y.; Kweon, D. H.; Lee, N. K.; Shin, Y. K. *Proc. Natl. Acad. Sci. (USA)* **2013**, *110*, 4+087.